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Evaluation of Diagnostic Performance of a Polymerase Chain Reaction for Detection of Canine Dirofilaria immitis

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Abstract : Diagnostic performance of polymerase chain reaction (PCR) for detecting Dirofilaria immitis in dogs was evaluated when no gold standard test was employed. An enzyme-linked immunosorbent assay test kit (SnapTM, IDEXX, USA) with unknown parameters was also employed. The sensitivity and specificity of the PCR from two-population model were estimated by using both maximum likelihood using expectation-maximization (EM) algorithm and Bayesian method, assuming conditional independence between the two tests. A total of 266 samples, 133 samples in each trial, were randomly retrieved from the heartworm database records during the year 2002-2004 in a university animal hospital. These data originated from the test results of military dogs which were brought for routine medical check-up or testing for heartworm infection. When combined 2 trials, sensitivity and specificity of the PCR was 96.4-96.7% and 97.6-98.8% in EM and 94.4-94.8% and 97.1-98% in Bayesian. There were no statistical differences between estimates. This finding indicates that the PCR assay could be useful screening tool for detecting heartworm antigen in dogs. This study was provided further evidences that Bayesian approach is an alternative approach to draw better inference about the performance of a new diagnostic test in case when either gold test is not available.

Key words : canine heartworm, PCR, diagnostic performance, Bayesian.

Introduction

Canine heartworm disease, due to infection with *Dirofilaria immitis*, is a potential zoonotic parasite transmitted by vector mosquitoes and occurs worldwide in tropical, sub-tropical and temperate zones (21). In Korea, human filariasis has almost been eradicated except in a very restricted coastal area, but the seroprevalence of *D. immitis* in dogs has been reported increasing to date with ranging from 9.6% to 40% (26). Ecological conditions such as temperature and relative humidity are known to be important for the intermediate host in the transmission of the parasite (24).

Commercial antigen test kits for diagnosing adult heartworm infection in dogs are available and the use of kits have risen primarily due mainly to improvements in diagnostic accuracy (sensitivity and specificity) and antimicrofilarial effects of commonly used macrolide preventatives (7). Although numerous reports evaluated or compared the sensitivity and specificity of various antigen test kits in dogs (3,5,6,9), selecting a kit for in-clinic use is a difficult task because many factors including practicality, speed, cost and ease of use must be considered. Of these factors, the single most important factor is the accuracy of the test. The sensitivity of a test is the probability that the test will correctly identify an infected dog. Likewise, the specificity is the probability that the test will correctly identify an uninfected dog (25). In clinical settings, it is more helpful to recognize of a test in terms of both the sensitivity and specificity, which are equally important.

The majority of test kits have limitations in use in that the sensitivity of these kits declines with all male or low female worm burdens. Furthermore, the evaluation of these test kits using sera from dogs that are heavily infected with heartworms is not likely reflective of cases seen in the typical veterinary practice. A polymerase chain reaction (PCR) has been used for detecting genomic DNA of the parasite in whole blood of infected dogs or mosquitoes (12,20,32). However, diagnostic performance of the PCR assay with field samples was not fully evaluated. The objective of this study was to estimate the sensitivity and specificity of the PCR for detection of *D. immitis* in dogs when the true disease state is unknown.

Practical illustration

Data

Heartworm database records (Kangwon National University) from May 2002 to September 2004 were used to determine diagnostic performance of the PCR. These data were from test results of blood samples referred from military shepherd dogs which were brought to an animal clinic for routine medical check-up or testing for heartworm infection. At the time of collection, all dogs were in-service with defined residence throughout the country. Dogs were included those with aged more than 8 months, not on chemoprophylaxis in last 12

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months and had not traveled out of in-service area. For analytical purposes, 2 study populations consisting 71 (random sample 1) and 62 records (random sample 2) were drawn from the database using random number function (RAND) in Statistical Analysis System (SAS, Cary, NC). This trial was repeated twice (trial 1 and 2) to check the stability of the estimates.

ELISA and PCR assay

Blood samples were collected from each dog by cephalic venipucture, and the serum samples were separated by centrifugation and kept refrigerated at −20°C until analysis. The circulating antigen released by adult female parasites of *D. immitis* was detected using an enzyme-linked immunosorbent assay (ELISA) test kit (Snap™, IDEXX, USA). For PCR assay, 100 microliters of whole blood was lysed in 0.1 M Tris-HCl (pH 8.0) containing 1% SDS, 0.1 M NaCl and 10 mM EDTA. Then the sample was treated with proteinase K (μl/ml) for 2 hr at 55°C. The DNA was extracted with phenol/chloroform, precipitated by ethanol, and then dissolved in TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). PCR amplification was performed using the DNA Thermo-Cycler (Perkin Elmer, USA). Specific primers and conditions were followed by the procedures described previously (20).

Estimation of parameters

The sensitivity and specificity of PCR was estimated by two statistical methods: maximum likelihood (ML) and Bayesian. The MLs through the Expectation-Maximization (EM) algorithm are a set of parameters that were most likely to have generated the observed data, and estimates was obtained by maximizing the likelihood function (15,27,31). Test results were cross-classified in a 2 × 2 table according to the status of each dog tested by the two tests, and six likelihood equations representing the probability of seeing the observed data in each cell conditional on the parameters were determined (Table 1): for each population, prevalence θ_1, θ_2, sensitivity Se_1, Se_2, specificity Sp_1, Sp_2. For example, the likelihood equation for cell "a" was determined as the sum of probabilities of two events: if the dog had been truly infected, then both tests give a true positive result and the conditional probability that the dog yield a test result in cell "a" is $\theta_1 Se_1 Se_2$. If the dog had been truly uninfected, then both tests give a false-positive result and the conditional probability that the dog yield a test result in cell "a" is $(1-\theta_1)(1-Sp_1)(1-Sp_2)$. Consequently, the overall probability of a dog yielding a test result in cell "a" is $[\theta_1 Se_1 Se_2]+[(1-\theta_1)(1-Sp_1)(1-Sp_2)]$. Likelihood equations for the remaining cells were derived similarly. A detailed explanation for the equation is described elsewhere (8,14,18,27,30).

Alternatively, Bayesian approach using Gibbs sampling which is based on the combined input from the likelihood and the joint prior was applied using a uniform (non-informative) prior: Uniform(0,1) = Beta(1,1). Detailed computation processes are given elsewhere (10,17,19,22). Conditional independence between the two tests was made. This implies that given that a dog is diseased or not, the probability of positive or negative outcomes for ELISA test is the same regardless of a known outcome for the PCR (13). It is assumed that the accuracy of both tests remains constant over different populations. A general discussion on this subject is provided by previous studies (4,19,28).

Table 1. Cross-classification of PCR result and ELISA kit for *Dirofilaria immitis* in the two dog populations and likelihood equations for a test result being in a given cell of the populations

ELISA result	Sample 1			Sample 2		
	Positive	Negative	Total	Positive	Negative	Total
Positive	a*	b	a + b	e	f	e + f
Negative	c	d	c + d	g	h	g + h

Likelihood equations#:

$a = \theta_1 Se_1 Se_2 + (1-\theta_1)(1-Sp_1)(1-Sp_2)$; $b = \theta_1 Se_1(1-Se_2) + (1-\theta_1)(1-Sp_1)Sp_2$

$c = \theta_1(1-Se_1)Se_2 + (1-\theta_1)Sp_1(1-Sp_2)$; $d = \theta_1(1-Se_1)(1-Se_2) + (1-\theta_1)Sp_1Sp_2$

$e = \theta_2 Se_1 Se_2 + (1-\theta_2)(1-Sp_1)(1-Sp_2)$; $f = \theta_2 Se_1(1-Se_2) + (1-\theta_2)(1-Sp_1)Sp_2$

$g = \theta_2(1-Se_1)Se_2 + (1-\theta_2)Sp_1(1-Sp_2)$; $h = \theta_2(1-Se_1)(1-Se_2) + (1-\theta_2)Sp_1Sp_2$

* Letters denote the number of sampled dog with the given result.

Parameters for ELISA and PCR in each population: $\grave{e}_1$, $\grave{e}_2$=prevalence; Se_1, Se_2=sensitivity; Sp_1, Sp_2 = specificity.

Table 2. Observed test results of PCR and ELISA for *Dirofilaria immitis* in the two populations of dogs

ELISA result	Sample 1			Sample 2		
	Positive	Negative	Total	Positive	Negative	Total
[Trial 1] Positive	11	0	11	18	1	19
Negative	1	59	60	1	42	43
[Trial 2] Positive	10	0	10	17	1	18
Negative	2	59	61	2	42	44

Table 3. Maximum likelihood estimates using Expectation-Maximization (EM) algorithm and Bayesian technique, for ELISA and PCR for detection of *Dirofilaria immitis* in dogs

Parameter*	Trial 1		Trial 2	
	EM algorithm	Bayesian (95% CI)	EM algorithm	Bayesian (95% CI)
θ_1 (%)	15.9	17.1 (9.2-26.6)	14.9	16.3 (8.6-25.9)
θ_2 (%)	31.5	31.6 (18.9-43.7)	30.6	31.0 (17.8-43.4)
Se_1 (%)	97.4	93.6 (81.2-99.7)	94.8	90.4 (75.0-99.5)
Se_2 (%)	96.7	94.8 (81.0-99.8)	96.4	94.4 (79.3-99.8)
Sp_1 (%)	100.0	98.6 (95.4-99.9)	100.0	98.6 (95.4-99.9)
Sp_2 (%)	98.8	98.0 (93.0-99.9)	97.6	97.1 (90.9-99.9)

* CI, Bayesian credible interval. Parameters for ELISA and PCR in each population: θ_1, θ_2 = prevalence; Se_1, Se_2 = sensitivity; Sp_1, Sp_2 = specificity.

Interpretation of test results

Test results by ELISA and PCR are shown in Table 2. The prevalence of *D. immitis* for EM and Bayesian approach was ranged 31.5-31.6% in trial 1 and 30.6-31.0% % in trial 2. In the first sample of trial 1, 12 samples were positive for PCR with 1 disagreement between the two tests, whereas 19 samples in the second sample were positive with 2 disagreements. In trial 2, 2 and 3 disagreements were observed for sample 1 and 2, respectively. Sensitivity and specificity of the PCR was quite similar in both trials: 94.8% and 98.0% for trial 1 and 94.4% and 97.1% for trial 2 (Table 3). The results of Bayesian were close to those of EM algorithm with no statistical difference at significance level of 0.05.

Discussion

Estimating the accuracy of a diagnostic test is a critical concern for many clinicians whenever new test is under evaluation. This study illustrated two-population model where two diagnostic tests with unknown diagnostic accuracy were simultaneously applied to individuals from two study populations with different prevalences of disease.

The ML approach can be subjected to produce biased results, if not taken into underlying assumptions, resulting in parameter estimates can be unstable or be produced very wide confidence intervals. Most simply, the sensitivities and specificities of the two tests (PCR and ELISA) are conditionally independent. The assumption can be considered reasonable because ELISA is based on detecting heartworm antigen released into the blood primarily by adult female worms, while PCR is based on detecting a specific DNA sequence. Vacek (28) showed that if conditional dependence exists between two tests, then classification errors for both tests will be substantially underestimated. An additional assumption is that each test has the same performances over different populations. In this study sensitivities and specificities in EM were quite similar to those of Bayesian, as seen in Table 3. On the other hand, in situations where prevalence or sensitivity is low, and limited sample size was used (14), the ML estimates may not converge. Addition of small number all cells may provide a solution to this problem, but this approach can introduce bias into the estimates. This was not problematic in this study and thus confidence intervals can be obtained.

In many clinical settings, there is no perfect test available to classify whether an animal is infected or not. When an imperfect test is applied to determine disease status, biases always are introduced into both measurements of test performance, and led to over-or under-estimates of a test's true capabilities (22,29). To deal with the situation where the sensitivity and specificity of a test are not precisely known alternative methods have been proposed and known to be useful tools (1,2,11,19,23). Of these, the Bayesian approach has been used to model a priori knowledge about unknown parameters and to combine this with the information contained in the likelihood based on observed data (10,16). In this study, the uniform prior distribution was used to the unknown parameters because of ease of calculation and flexibility. Further studies need to be performed to determine the effect of prior probability distributions for the six parameters of interest on the posterior distributions.

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개 심장사상충을 진단하기 위한 중합연쇄반응검사 (PCR)의 진단적 특성 평가

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요 약 : 본 연구는 개에서 심장사상충을 검출하기 위하여 표준검사를 적용하지 않은 상황에서 중합연쇄반응검사 (PCR)의 진단 능력을 평가하였다. 효소면역검사법 (ELISA)과 PCR 검사를 동시에 사용한 경우 PCR 검사의 민감도와 특이도는 두 검사의 조건부 독립을 가정한 상태에서expectation-maximization (EM) 알고리즘을 이용한 최대우도법과 Bayesian 기법으로 두 집단 검사 모형으로 분석하였다. 2002-2004년 기간 중 심장사상충검사 결과를 기록한 의무기록에서 무작위로 266개 결과를 추출하여 133개씩 2회의 시험으로 배치하였다. 2회의 분석결과를 종합할 때 EM 알고리즘에서 PCR 검사의 민감도와 특이도는 각각 96.4-96.7%와 97.6-98.8%, Bayesian 기법에서는 94.4-94.8%와 97.1-98%로 추정되었다. PCR 검사는 심장사상충을 스크리닝하는 도구로 유용하며, 표준검사를 적용하지 않은 상황에서 진단검사의 특성을 추론하는 방법으로 Bayesian 기법은 매우 유용함을 확인하였다.

주요어 : 개 심장사상충, 중합연쇄반응검사, 진단능력, Bayesian